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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

To test our hypothesis that that the HA binding peptide (HABP/tachyplesin) may be a new anti-neurofibromatosis agent, the peptide were chemically synthesized in a large scale and its bioactivity on NF cells was examined and underlying mechanism was explored. The results showed that 1) HABP can significantly change the morphology of treated ST88-14 and STS26T NF cells and inhibit their proliferation or colony formation in a dose-dependent manner; 2) HA binding peptide binds to Bcl-2/Bcl-x_L and induces apoptosis, which may be one of the mechanisms by which HABP inhibits ST88-14 and STS26T NF cells; 3) HA binding peptide is capable of reducing the level of phosphorylated ERK1; 4) HABP reduces the level of cell cycle related molecules, such as cyclin B1 and cdc 2; 5) HABP/tachyplesin binds to C1q on cell surface, activate the classical complement cascade, since it triggered several down-stream events including the cleavage and deposition of C4 and C3 and the formation of C5b-9 and destroy the integrity of tumor cells; 6) the HABP/tachyplesin exerts anti-tumor effect on NF cells both *in vitro* and *in vivo*.

Since the HABP /tachyplesin is a simple biological peptide without any toxicity, it might have the potential to be used in anti-NF. It is worth to further pursue this project for developing a new agent to against the cell growth of neurofibromatosis.

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INTRODUCTION

Neurofibromatosis Type 1 (NF1) is one of the most common autosomal dominant genetic disorders in humans (1-3). Clinically, NF1 is a transmitted cancer predisposing syndrome that generally results in multiple peripheral nerve tumors as the most prominent feature (1-3). Molecular and biochemical analysis of tumors from patients with NF1 indicates that there is a high frequent loss of the normal NF1 gene allele (3) and mutations in the gene (5).

The NF1 gene, responsible for this disease, encodes a large cytoplasmic protein called neurofibromin. Similar to p53, neurofibromin is regarded as a tumor suppressor, since it plays a major role in the negative regulation of Ras activity which is crucial in cell survival signal transduction pathway. The loss of neurofibromin leads to the accumulation of hyperactive Ras-GTP due to a reduced conversion of active Ras-GTP to inactive Ras-GDP, which turns on uncontrolled mitogenic signals in the nucleus (2, 4-7). Therefore, NF1 can be regarded as a disease resulting from the disruption of the balance between cell proliferation and apoptosis (8, 9). In other words, NF1 cells gain immortality due to their over-proliferation and defective apoptosis.

Based on the information of the underlying molecular mechanism of NF1, several new therapeutic approaches for NF1 have been proposed, such as down-regulation of the Ras activity or up-regulation of the apoptosis (10-12) to reduce the proliferation of NF1 cells. For example, researchers in Bristol-Myers Squibb Pharmaceutical Research Institute have used BMS-18651 (an inhibitor of farnesyltransferase, FT) to block the FT-mediated post-translational farnesylation of Ras proteins, which is required for Ras function. They found that the inhibition of functional maturation of Ras could reduce the malignant growth properties of ST88-14, a cell line established from malignant Schwannoma of a NF1 patient (11). This represents a promising a new treatment of NF1.

Recently, the regulation of apoptosis has been utilized for the control of cell growth and the treatment of tumors (14-18). It has been demonstrated that the up-regulation of apoptosis by wide-type p53 can inhibit the growth of a variety type of tumors (19-20) that have the lost or mutant p53. The specific targeting of p53 to hsp 70 on mitochondria can induce apoptosis, and thereby, reduce uncontrolled cell proliferation (20-21). Similarly, up-regulation of apoptotic-promoting proteins, such as the caspase family or Fas and Fas ligand (22-24), or down-regulation of apoptotic inhibitors, such as bcl2, will also reduce the growth of tumor cells (25-26).

Alone these lines, we have been investigating naturally-existing substances that can induce apoptosis and inhibit the tumor cell growth but are not toxic to normal cells. Recently, we have found that hyaluronan (HA) binding proteins (HABPs) possess anti-tumor properties.

The rational for our pilot studies was based upon the following facts. **1)** Proteins that can bind to HA such as the soluble forms of CD44 and RHAMM can inhibit tumor growth and/or metastasis (27-29). **2)** Fragments of proteins that contain HA binding domain, such as endostatin (fragment of collagen XVIII), angiostatin (fragment of plasminogen), and hemopexin-like domain of metalloproteinase also possess potent anti-tumor activity (30-33). **3)** For more than a decade, the powder or extracts from shark cartilage have been widely used as alternative medicine by cancer patients in USA, Europe and Asia. In some patients, this substance did exhibit anti-tumor effects. Cartilage contains large amounts of HA binding proteins (HABP). It is possible that the anti-tumor effect of shark cartilage achieved in some patients is due to a small amount of HABP passing through impaired intestinal mucosa of these individuals (34-42). And **4)** several proteins purified from the cartilage, a HA-rich tissue, have been found to have the anti-tumor effect (43-48).

In this study, we propose to test our hypothesis that HA binding peptide may be a new anti-neurofibromatosis agent via inducing apoptosis. For this, we are focusing on three aims: **1):** To examine the anti-tumor effect of synthetic targeted HA binding peptide on malignant neurofibromatosis cells; **2):** To examine the anti-tumor effect of genetically expressed targeted HA binding peptide; and **3)**: To examine the effect of targeted HA binding peptide on molecules involved in apoptosis.

BODY

In neurofibromatosis**,** the loss or mutation of NF1 gene is the molecular error responsible for the phenotype. The defective neurofibromin leads to accumulation of hyperactive Ras-GTP, which make the mitogenic signals in NF1 cells "turn on" all the times, and the cells respond by proliferating. We speculated that HABP might be able to induce apoptosis and to reduce the growth of NF1 cells.

We have performed many experiments in the grant funded 4 years and generated very meaningful data that lead us to define the anti-tumor effect of HABP and its underlying mechanisms.

The data are summarized in the following:

1. Synthesis and characterization of HA binding peptide:

The HA binding peptide (HABP) that we have proposed to test consists of 17 residues of amino acid: **K**WCF**R**VCY**R**GICY**RR**C**R,** which consists of two domains of B[X7]B: **K**WCF**R**VCY**R** and **R**GICY**RR**C**R**. Due to the four residues of cystine (C), this peptide forms two disulfide linkage, which exposes all six positively charged basic amino acids on its surface, allowing effective binding to its ligand or target. Furthermore, it confers stability to the peptide at low pH and high temperature, which makes it easy to manipulate and gives it a long shelf-life.

Due to its short sequence of amino acid, this HABP is relatively easy to make it by chemical synthesis. If HA binding peptide does exert a potent anti-tumor effect on neurofibromatosis, then practically it can be produced in a large quantity for the clinical trial.

The HA binding peptide and its scramble control peptide which has the same amount of amino acid were submitted to Genemed Synthesis Inc. (South San Francisco, CA) to synthesize chemically for the quantity of one gram. To avoid the enzyme degradation, the N-terminal were acetylated and the C-terminal amidated. The result of mass spectrophotometer analysis indicated that the synthetic peptide had molecular weight of 2309 Dalton with a high purity.

To determine if this synthetic HA binding peptide has bioactivity, the binding assay was performed. The peptide was mixed with ^{3}H-HA, incubated for 2 hours and then applied to a nitrocellulose membrane using a dot blot apparatus. The free ^{3}H-HA was washed away with phosphate saline buffer, and then the membrane with retained peptide-^{3}H-HA complex was cut out, emerged in scintillation fluid and the radioactivity was determined with a β-counter. The results (**Fig. 1**) showed that this synthetic targeted peptide had strong HA binding properties which could be blocked with a 50 fold excess of cold HA, while the scramble control peptide had very little background binding

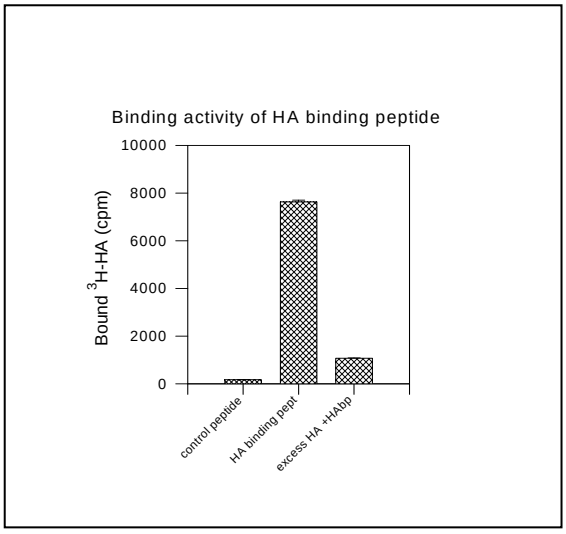


Fig 1. HA Binding activity of HA binding peptide. Fifty μg of targeted peptide was incubated with 20 μl of 100 μg /ml of ^{3}H-HA (5 x 105 cpm/μg HA) for 2 hours and loaded onto nitrocellulose membrane on a dot blot device. The free ^{3}H-HA was washed away with PBS, and then the membrane with retained peptide-^{3}H-HA complex was cut out, emerged in scintillation fluid and the radioactivity was counted with a β-counter. The targeted peptide binds to HA.

The successful synthesis of HA binding peptide provided material for its anti-neurofibromatosis function study.

2. Effect of synthetic HABP on neurofibromatosis cells

A) Effect of synthetic HA binding peptide on ST88-14 NF1 cells

Initially, we used HA binding peptide to treat the human ST88-14 NF1 cells and endothelial cells (human umbilical vein endothelial cells, HUVEC) in anchorage-dependent culture. It took less than 24 hours to cause a visible morphological change in HA binding peptide treated cells. The cells treated with HA binding peptide looked very sick, appearing rounded, condensed and detached (**Fig. 2B and D**), much different from those treated with the control peptide (**Fig. 2A and C**). HA binding peptide seems have strong effect on cell viability. This effect of HA binding peptide on ST88-14 NF1 cells was comparable with Taxol, a most commonly used anti-tumor drug, which was used as positive control in the experiments (**Fig. 3**).

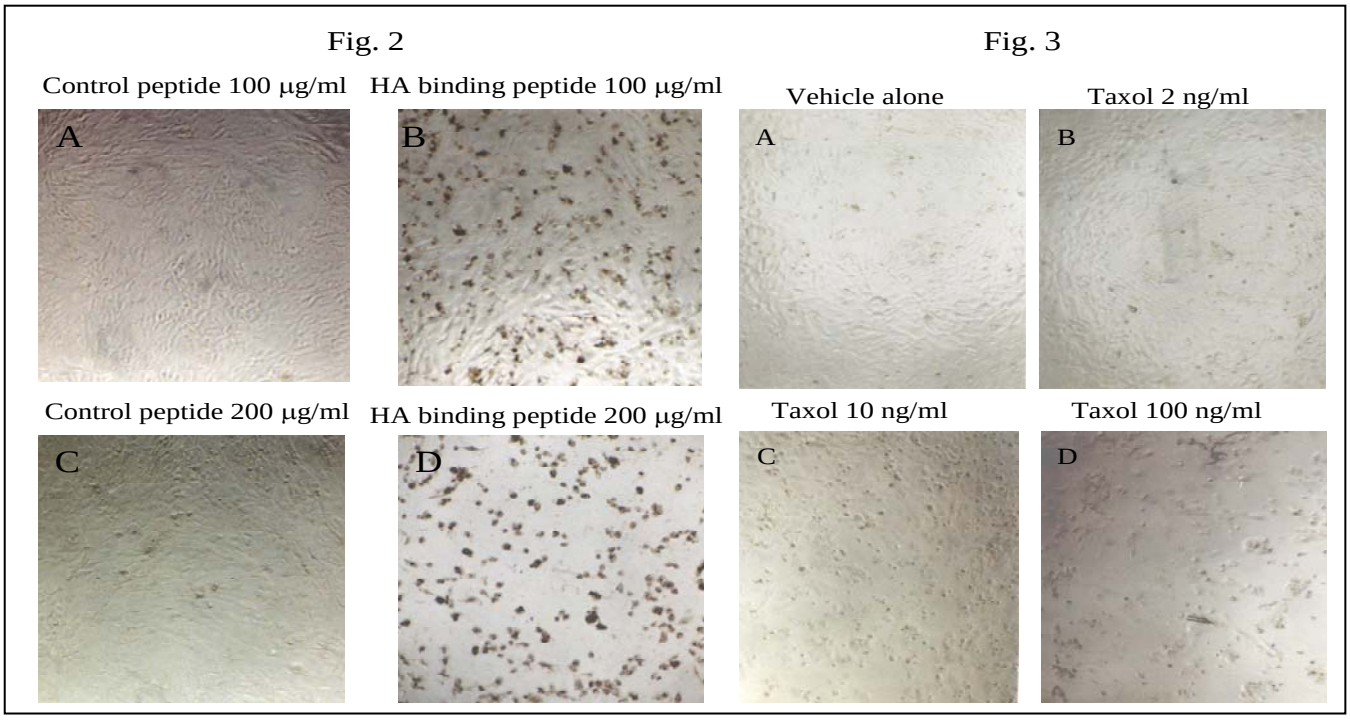


Fig 2 and 3. Effects of HABP and Taxol on ST88-14 cells. The cells were treated with indicated agents for 24 hours and then the pictures of altered morphology were taken under reverse microscopy.

To quantitatively measure the effect of HA binding peptide on cell proliferation, ^{3}H-thymidine (^{3}H-TdR) incorporation assay was carried out. The ST88-14 NF1 cells were cultured in 96 well plates and treated with different doses (25, 50, 100 or 200 μg/ml) of HA binding peptide for 24 hours, and then treated with ^{3}H-TdR to detect cell proliferation. The results showed that the HA binding peptide had a strong inhibitory effect on ST88-14 NF1 cells in a dose dependent manner ($P<0.01$, **Fig. 4**). Furthermore, the proliferation of HUVEC induced by FGF2 was also inhibited by HA binding peptide ($P<0.01$, **Fig. 5**). These inhibitory effects were very reproducible.

Importantly, when tested on non-tumorigenic, immobilized normal cell lines (Cos 7 and NIH 3T3), the inhibitory rate was much lower than that either on the ST88-14 cells or on the proliferating endothelial cells, indicating that HA binding peptide might be preferentially targeting tumor cells (data not shown).

Fig 4

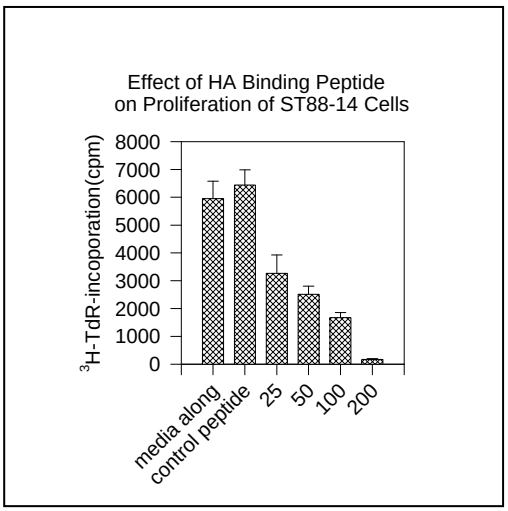


Fig 5

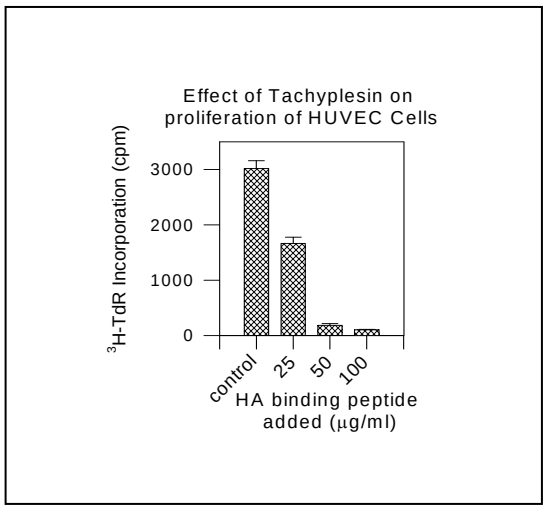


Fig 4 and 5. Effects of HABP on ST88-14 cells and HUVEC cells. The cells cultured in 96 well plate were treated with HA binding peptide at different concentrations for 24 hours and then traced with ^{3}H-TdR for proliferation. The incorporated ^{3}H-TdR was counted with β-counter.

B) Effect of synthetic HABP on malignant neurofibromatosis STS26T cells

To explore if the anti-NF effect of HABP is universal, another human NF cell line was tested.

a) Effects of HABP on anchorage dependent growth of STS26T cells: The STS26T cells were cultured in 96 well plate, treated with 50 ug/ml of HABP, and examined their morphology alterations under microscope with time. There were large amount of HABP treated cells appeared to undergoing cell death and the cell number was significantly lower than that of vehicle control (**Fig 6**). The MTT assay for cell proliferation and viability showed that the HABP treated cells were much lower than the vehicle control (**Fig 6B**), suggesting the STS26T cells were sensitive to killing effect of HABP.

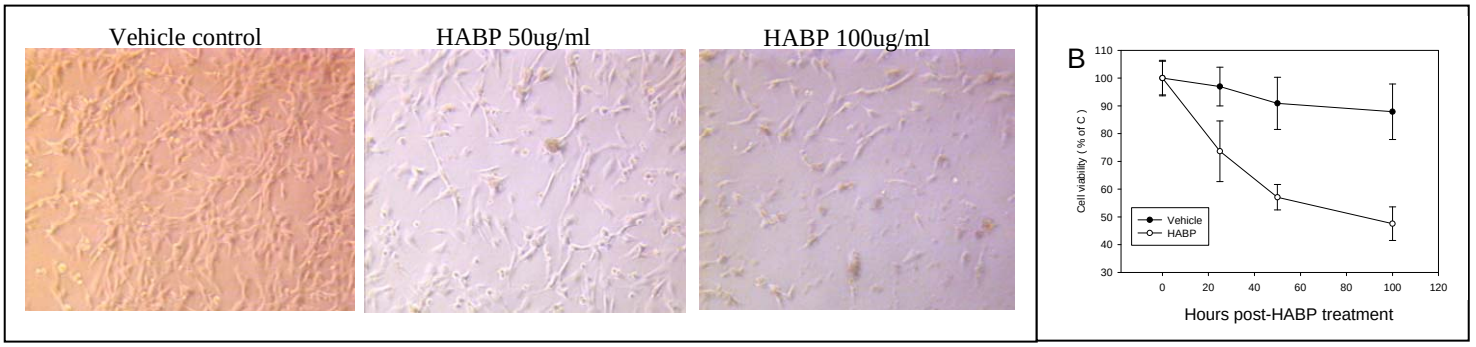


Fig 6. Effects of HABP on anchorage dependent growth of STS26T cells. The cells cultured in 96 well plate were treated with HABP at different concentrations. **A)** The cell pictures were taken 4 days after treatment. **B)** the cell viability was measured with MTT assay at indicated time points and the survival rate relative to vehicle control (as 100%) was calculated. The difference between the vehicle alone and HABP treatment groups were statistically significant ($P<0.05$).

b) Effects of HABP on colony formation of STS26T cells: Colony formation in soft agar is a good index for tumor malignance *in vivo*. Twenty thousands of STS26T cells in 0.36% soft agar with 10% FBS-IMEM and 0 (as control), 50 or 100 ug/ml of HABP were added on the top of a layer of 0.6% bottom agar. At day 20, the colonies with cell number >50 were counted. The results (**Fig 7 A-C**) showed that the number

of colonies in HABP treated groups were much lower than that in vehicle group, and this difference was statistically significant (P<0.05, **Fig 7D**).

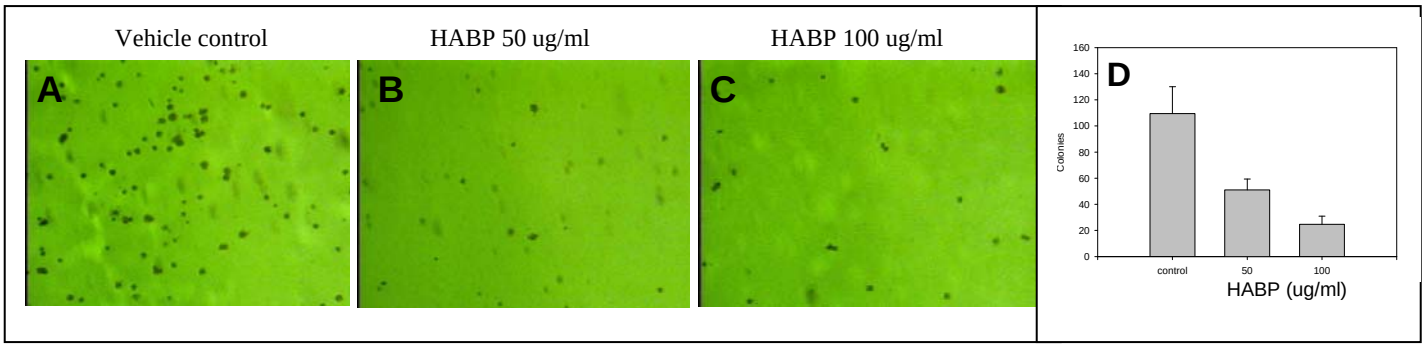


Fig 7. Effects of HABP on colony formation of STS26T cells. The STS26T cells grown in soft agar were pictured and counted for the number of colonies (>50 cells) on day 20 after seeded. The difference between the vehicle alone and HABP treatment groups were statistically significant (P<0.05).

These results indicated that the HABP did possess potent anti-tumor activity in NF cells.

3. Molecular mechanisms underlying the anti-tumor effect of HABP

To explore the underlying mechanism by which HA binding peptide exert its anti-tumor activity, we have carried out the studies focused on the molecules involved in cell apoptosis and proliferation. The results indicate that HA binding peptide could bind to anti-apoptosis molecules, such as Bcl-2/ Bcl-x_L. It could also reduce cyclin B1, Cdc-2 and phosphorylated erk. The data are showed as following:

A) Binding of HA binding peptide to Bcl-2

Bcl-2/ Bcl-x_L, the critical anti-apoptotic molecules, are anchored on membranes and may form a large macromolecular structure or lattice. This three-dimensional configuration is stabilized with the binding of Apaf-1, an adaptor for caspase 9. When a pro-apoptotic member of the Bcl-2 family binds to an anti-apoptotic member, the lattice conformation alters, forms ion-conducting channels [49-51], and releases Apaf-1 and cytochrome c from mitochondria into the cytosol. The entire lattice becomes conducive to caspase activation and apoptosis induction.

Obviously, the functional blockade of Bcl-2 / Bcl-x_L could restore the apoptotic process, and thereby, could inhibit the uncontrolled proliferation of ST88-14 NF1 cells.

To determine if HA binding peptide acts on Bcl-2/Bcl-x_L, we performed two types of experiments. First, an **ELISA-like binding assay** was performed. The HA binding peptide or control peptide were coated on maleic anhydride treated plates (special for coating of peptide, Micro membranes Inc.). After blocking, different concentrations of purified, human recombinant Bcl-2 protein (Sanda Cruz, Inc) were added to plate and incubated at room temperature for one hour. After wash, the anti-Bcl-2 was added for one hour followed by peroxidase labeled second antibody and then substrate. While there was no binding in control peptide, the HA binding peptide bound strongly to Bcl-2 in a dose-dependent fashion (**Fig. 8**).

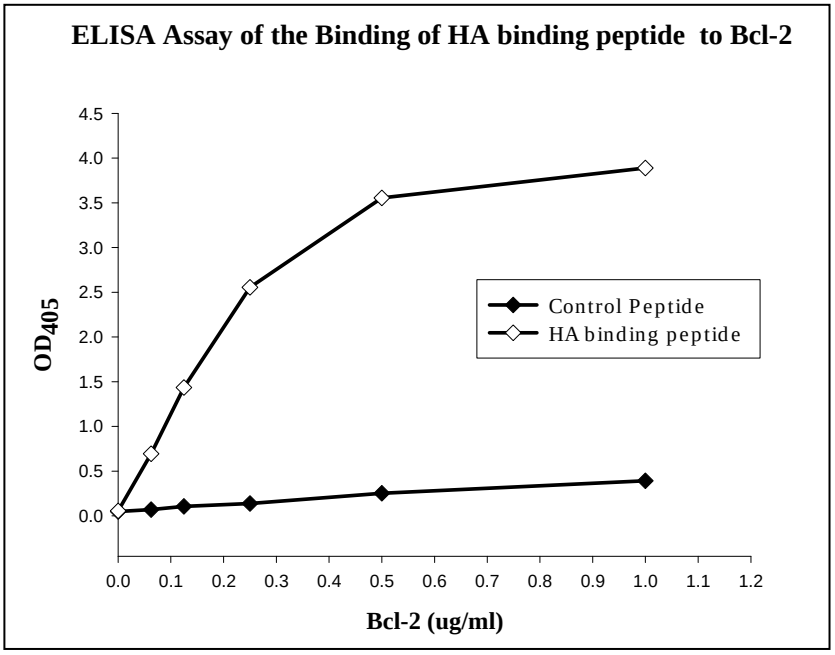
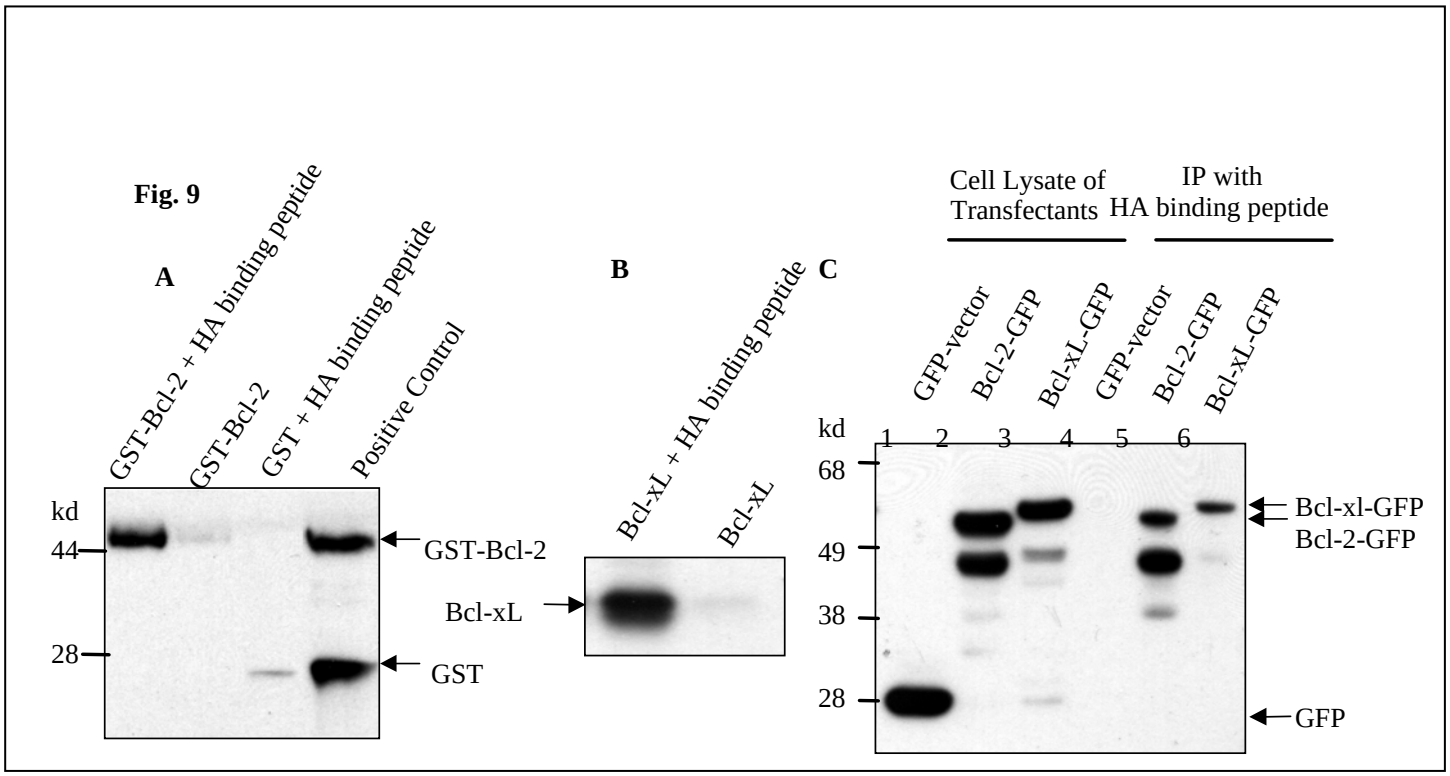


Fig. 8 Binding of HA binding peptide to Bcl-2. The High-Bond ELISA plates were coated with 200 μl of 5 μg/ml HA binding peptide or control peptide. After blocking each well with 5% BAS in TBS-T (10 mM Tris-Cl pH 7.4, 300 mM NaCl, 0.2%Tween-20), 100 μl of a series concentration of Bcl-2 (0 – 1.0 μg/ml) were added to each well of the plates and incubated at RT for one hour followed by incubation of anti-Bcl-2 antibody and then a second antibody-HRP. The plates are again washed with TBS-T and then each well was added 100 μl of a peroxidsae substrate consisting 0.03% H2O2, 0.5 mg/ml 2,2' azinobis (3-ethylbenzthiazoline sulfonic acid) in 0.1M sodium citrate pH 4.2. After 30 min, the OD_{405} will be determined using an ELISA reader.

To confirm if this is a true phenomenon and if HA binding peptide can also bind to Bcl-$x_{L,}$ the immuno-precipitation and Western blotting was performed ***in vitro***. 0.2 μg of purified GST-Bcl-2 fusion protein (Santa Cruz) or Bcl-x_L protein (expressed and purified from *E. coli*) or other GST fusion protein (as control for specificity) were incubated with (as test) or without (as control) 0.2 μg of biotinylaed HA binding peptide in 500 μl of TBS-T buffer (300 mM NaCl, 1 mM EDTA, 20 mM. Tris [pH 8.0], 0.2% Tween 20) at 4°C. One hour later, each of the binding reactions was mixed with 20 μl of 1:1 slurry of strepavidin-sepharose beads and incubated overnight at 4°C with gentle agitation. The beads were washed three times with 1 ml of TBS-T. The bound proteins were eluted in SDS-loading buffer, electrophoresized and transferred to nitrocellulose membrane followed by staining with anti-GST tag (for Bcl-2) or anti- Bcl-x_L secondary antibodies labeled with peroxidase and enhanced chemiluminescent (ECL) detection. The result (**Fig. 9A**) was very convincing: a major band was pulled down by strepavidin-sepharose beads in the mixture of GST-Bcl-2 and biotin-HA binding peptide (lane 1), indicating that the GST-Bcl-2 physically binds to HA binding peptide . This binding specificity was confirmed that no major band can be detected when **only** present of GST-Bcl-2 **without** biotin-HA binding peptide (lane 2) GST tagged **other protein** with biotin-HA binding peptide (lane 3). The majority of GST-Bcl-2 in the test tube was bound to biotin-HA binding peptide (lane 1) as compared to the loading control (lane 4). To our amazing, similar situation occurred in mixture of **Bcl-x_L with biotin-HA binding peptide** (**Fig. 9B**), in which there was a clear band in lane 1 while no band could be detected without addition of biotin-HA binding peptide (lane 2).

Does this happen ***in vivo*** in a natural condition? The 293T cells were transiently transfected pCMV vector carrying with Bcl-2 or Bcl-x_L cDNA fused at N-terminal of Green Fluorescence Protein (GFP) or with only GFP (as a control). Twelve hours later, cells were incubated with biotin-HA binding peptide for 3 hours and 1.0 ml of cell lysates were mixed with strepavidin-sepharose beads and incubated overnight at 4°C. The bound protein were eluted in SDS loading buffer analyzed in Western blotting with anti-GFP. The results showed that although all the cells (in lanes 4-5 of **Fig. 9C**) received biotin-HA binding peptide treatment, the strepavidin-sepharose beads could pulled down GFP fusion proteins only in Bcl-2-GFP or Bcl-x_L-GFP transfected cells (**Fig. 9C Lane 5, 6)** not in vector alone transfectants (**Fig. 9C Lane 4**). The lysates of transfected cells (**Fig. 9C Lanes 1-3**) had multi-bands, indicating a degradation of expressed Bcl-

2-GFP or Bcl-x_L-GFP fusion protein. We noticed that a truncated Bcl-2 (lower band in **Fig. 9C, Lane 5**) had a higher binding affinity to HA binding peptide than intact Bcl-2 (up band in **Fig. 9C, Lane 5**).



Figs. 9. The binding of HA binding peptide to Bcl-2 and Bcl-x_L: For ***in vitro* binding assay**, 0.2 μg of GST-Bcl-2 fusion protein or Bcl-x_L protein were incubated with or without 0.2 μg of biotin-HA binding peptide and incubated overnight with strepavidin-sepharose beads, followed by wash, elution and Western blotting with anti GST tag (for Bcl-2) or anti- Bcl-x_L, and ECL detection (**A and B**). **For *in vivo* co-immunoprecipitation**, 293T cells in 100 mm dishes were transiently transfected pCMV vector with Bcl-2 or Bcl-xL cDNA fused at N-terminal of GFP or with only GFP as a control. Twelve hours later, cells were incubated with biotin-HA binding peptide for 3 hours and harvested in 1.5 ml of lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM. Tris [pH 8.0], 0.5% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1mM PMSF). One ml of cell lysates were mixed with strepavidin-sepharose beads and incubated overnight at 4°C. The beads were washed, eluted in 30 ul of SDS loading buffer. 10 μl of each transfected cell lysates and the immunoprecipitated sample were analyzed in Western blotting using anti-GFP antibody and ECL detection. (**C**).

These *in vitro* and *in vivo* data indicate that the inhibitory effect of HA binding peptide on ST88-14 NF1 cells may due to the binding of HA binding peptide to Bcl-2/Bcl-x_L, which blocks their anti-apoptosis function, leading to enhanced programmed death of NF cells.

B) HABP binds to Bcl-2 *in vivo* and induces apoptosis.

It has been proved that Bcl-2/Bcl-x_L, the critical anti-apoptotic molecules, are anchored on membranes and may form a large macromolecular structure or lattice, which stabilizes the membrane of mitochondria and prevents the cells from apoptosis. The functional blockade of Bcl-2 / Bcl-x_L could restore the apoptotic process, and thereby, could inhibit the uncontrolled proliferation of NF1 cells.

In the first year, we were exciting about out findings that: **1)** HABP could bind to Bcl-2/Bcl-x_L as assayed with ELISA like system; and **2)** HABP could interact with recombinant Bcl-2 *in vitro*.

We then wanted to see if this interaction could occur *in vivo.* To determine this, the tumor cells cultured in 100 mm dishes were first transfected with mammalian expression vector containing cDNA cording for Bcl-2 with GFP tag for 24 hours, and then incubated with biotinylated HABP or biotinylated control peptide for 3 hours and harvested in 1.5 ml of lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM. Tris-HCl pH 8.0, 0.5% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1mM PMSF). One ml of cell lysates were mixed with strepavidin-sepharose beads and incubated overnight at 4°C. The beads were washed to get rid of miscellaneous protein, and then eluted with 30 μl of SDS loading buffer and boiling. Ten μl of transfected cell lysate was used as control. The samples were analyzed in Western blotting using anti-Bcl-2 antibody to detect the complex of HABP-Bcl-2-GFP.

The result (**Fig 10**) showed that the HABP could bind to Bcl-2 *in vivo* system.

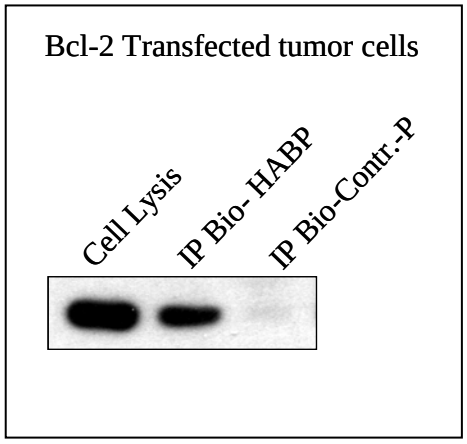


Fig 10. HABP binds to Bcl-2 *in vivo* system. The tumor cells cultured in 100 mm dishes were first transfected with mammalian expression vector containing cDNA cording for Bcl-2 with GFP tag for 24 hours, and then incubated with biotinylated HABP or biotinylated control peptide. The cell lysates were mixed with strepavidin-sepharose beads. The bound HABP-Bcl-2-GFP complex was detected using Western blotting with anti- Bcl-2 antibody.

Then, we wanted to examine if the interaction of HABP with Bcl-2 resulted in an apoptosis, since the critical anti-apoptotic molecule was disrupted. For this, tumor cells were treated with HABP or control peptide at a dose of 100 μg/ml. Twenty-four hours later, the DNA was harvested and subjected to the DNA ladder analysis.

The result (**Fig 11**) demonstrated that HABP indeed induced the apoptosis as determined by the DNA ladder analysis, a golden standard for the apoptosis.

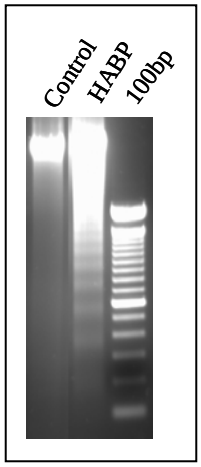


Fig 11. HABP induces apoptosis. The tumor cells were treated with HABP or control peptide at a dose of 100 μg/ml for 24 hour and then the DNA was harvested and subjected to the DNA ladder analysis.

The HABP-induced apoptosis was also studied in the STS26T cells using Annexin V/PI staining and sub-G1 cell population analysis with flow cytometry. The cells were treated with 0, 50 or 100 ug/ml of HABP for 3 days and assayed. The results showed that Annexin V/PI positive staining cells were increased in HABP treated group compared to vehicle group (25-37% v.s. 7%, **Fig 12**). Similarly, the sub-G1 population was increased in HABP treated group compared to vehicle group (20.8-29% v.s. 9.5%, **Fig 13**). All these data demonstrated that apoptosis was triggered by HABP.

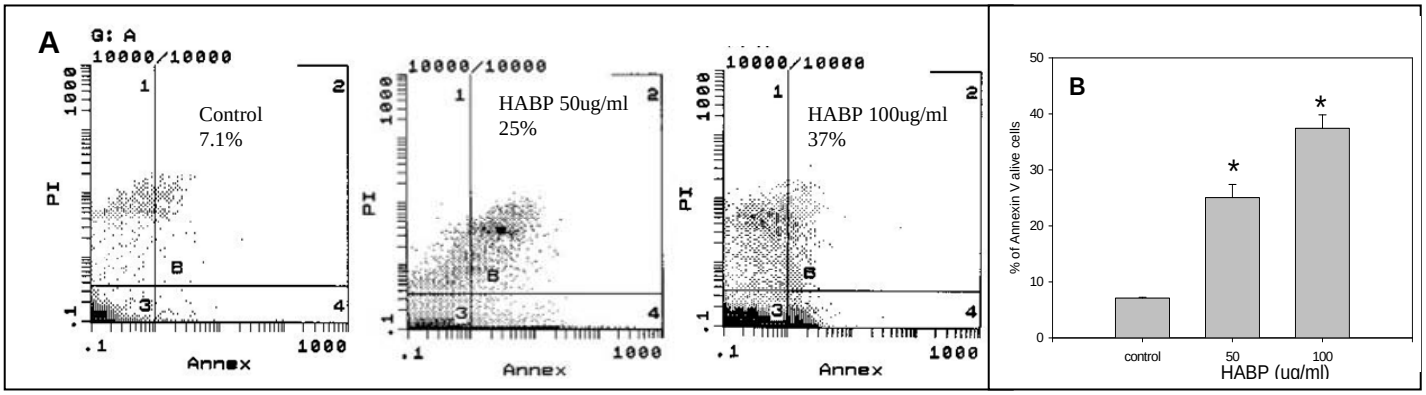


Fig 12. HABP triggered apoptosis as assessed with Annexin V/PI staining. The STS26T NF cells were treated with HABP at a dose of 50 or 100 ug/ml or vehicle alone for 3 days, stained with Annexin V/PI, and assayed with FCM.

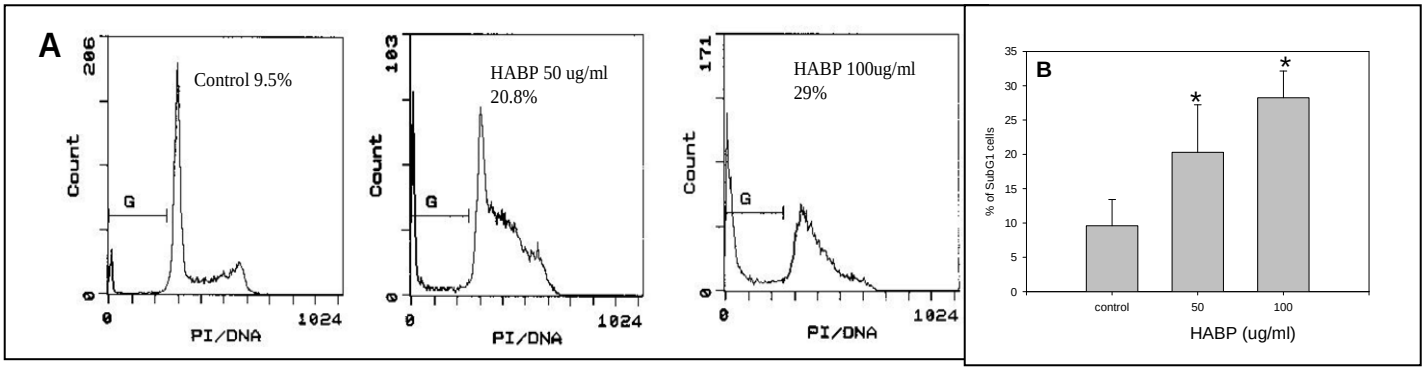


Fig 13. HABP triggered apoptosis as assessed with the sub-G1 population analysis. The STS26T NF cells were treated with HABP at a dose of 50 or 100 ug/ml or vehicle alone for 3 days, stained with PI, and assessed for sub-G1 population with FCM.

What is the underlying mechanism of these apoptosis phenomena? Why the binding of HABP could induce apoptosis. We speculate that HABP acts in a fashion similar to pro-apoptotic molecule in Bcl-2 family, such as such as Bim, Bid, Bak, Bad, Bax, Bcl-xs, Blk, Bnip3, Bik and Hrk (37-39). To see if this is the case, we searched the existing database for the similarity between the HABP and pro-apoptotic molecules.

The HABP consists of 17 residues of amino acid: **K**WCF**R**VCY**R**GICY**RR**C**R,** which contains two typical $BX_{6-7}B$ motifs, which is six to seven neutral amino acids franked by two basic amino acids (R: arginine; K: lysine; H: histine). Interestingly, this $BX_{6-7}B$ motif exists in all the pro-apoptotic Bcl-2 family proteins examined (**Table 1**). Some of them have more than one $BX_{6-7}B$ motif. Whether this similarity confers the HABP with the ability to bind to Bcl-2 will be further investigated.

Table 1. The similarity of HABP to pro-apoptosis proteins in BCL-2 Family ($BX_{6-7}B$ motif)

HABP:	**K**WCF**R**VCY**R**GICY**RR**C**R**		
Bim_L	**H**P**R**MVIL**R**LL**R**YIV**R**LVW**R**M**H**		
Bim_S	**R**FIF**R**LVW**RR**H		
Bid	**R**SS**H**S**R**LG**R**	**R**TYV**R**SLA**R**	**KK**VAS**H**TPSLL**R**
Bak	**R**LAL **H**VYQ**H**	**HH**CIA**R**W IAQ**R**	
Bad	**KK**GLP**R**P**K**	**R**YG**R**EL**RR**	**R**QSSSWT**R**
Bax	**K**LVL**K**ALCT**K**	**KK**LSECL**KR**	
Bcl-xS	**RK**GQE**R**FN**R**	**H**SSSLDA**R**	
Blk	**K**NNM**K**VAI**K**TL**K**	**R**QLLAPIN**K**	
	RQSL**R**LV**RK**	**K**GAFSLSV**K**	**R**WFF**R**SQG**RK**
Bnip3	**KH**P**KR**TATLSM**R**NTSVM**KK**		
Bik	**K**ENIM**R**FW**R**		
Hrk	**H**Q**R**TMW**RRR**ARS**RR**		

The motif or structure similarity of HABP with pro-apoptotic proteins in Bcl-2 may lay down the foundation for HABP being a novel death regulator for NF1.

C) HA binding peptide reduces the level of phosphorylated ERK1.

Since the loss of neurofibromin leads to the accumulation of hyperactive Ras-GTP due to a reduced conversion of active Ras-GTP to inactive Ras-GDP, the accumulation of hyperactive Ras-GTP results in a constitutive mitogenic signaling of cell growth (2, 4-7). The Ras signaling has been well identified as Ras---Raf---MRK---ERK/MARK transduction process.

It is obvious that signal through the RAS- ERK /MAPK pathway, phosphorylated ERK can be used as an indicator of when and where signaling is active.

Base on this fact of molecular error in NF1, we would like to see if the HABP can inhibit the ERK, which is the down-steam kinase responsible for further amplification of the hyperactive Ras-GTP function in NF1. For this, the ST88-14 cells, a typical line of NF1 cells, were plated in 100 mm dishes and treated with 100 μg/ml of control peptide or HABP (synthesized by experts of organic chemistry in Genemed Inc.). The cells were incubated with peptides for 24 hours and the cells were washed and harvested in lysis buffer (10 mM potassium phosphate at pH 7.5, 1 mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 0.5% Triton X-100, 0.1% sodium deoxycholate, 1 mM magnesium chloride and 2 mM DTT). The protein concentration was determined with BCA method (PIERCE Inc). Thirty μg of lysate protein was load in 10% SDS-PAGE for electrophoresis. After transferring to the nitrocellulose membrane and blocking with 3% BSA-PBS, the total ERK protein and phosphorylated ERK (the functional form of ERK) were detected with anti-ERK or anti-phosphorylated ERK, respectively. The result (**Fig 14**) showed that while the total ERK was not affected by the treatment of ST88-14 cells with HABP, the phosphorylated ERK was reduced.

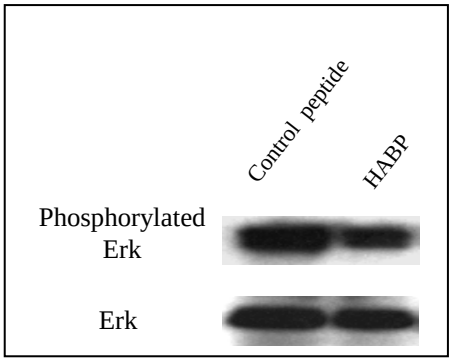


Fig 14. Blocking of phosphorylation of ERK with HABP. The ST88-14 cells were plated in 100 mm dishes and treated with 100 μg/ml of control peptide or HABP for 24 hours. The cell lysate was subjected to Western blot analysis. The result showed that while the total ERK was not affected by the treatment of ST88-14 cells with HABP, the phosphorylated ERK was reduced.

This data suggests that the HABP is capable of blocking the down-steam signaling of hyperactive Ras-GTP, the molecular error in NF1.

D) HA binding peptide reduces the level of cell cycle related molecules

The uncontrolled growth is one of the characteristics of NF1, which reflects a active process of cell cycle. The cyclins and their kinases (cdc) are the actual molecules that control the cell cycle. To determine the effect of HABP on the cyclins and their cdc, the Western blotting analysis was performed after the cells were exposed to HABP for 24 hours. The results (**Fig 15**) showed that while cyclin D1 was unchanged, the cyclin B1 and cdc2 were greatly reduced at the protein level.

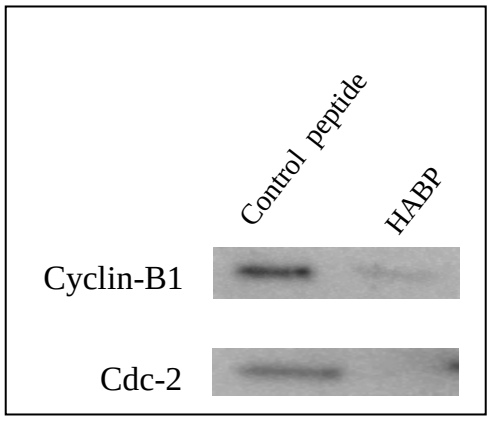


Fig 15. HABP Inhibition of cyclin B1 and cdc2. The tumor cells were treated with 100 μg/ml of control peptide or HABP for 24 hours. The cell lysate was subjected to Western blot analysis. The result showed that the levels of cyclin B1 and cdc2 were greatly reduced.

The above data indicates that the HABP interacts with functional proteins at both the RAS-ERK /MAPK pathway and the cell cycle related cyclin B1 and cdc2. This multi-levels control is favorable for HABP as a potential anti-NF1 agent.

E) HABP/tachyplesin anti-tumor effect is related with activation of complement cell killing pathway: We have demonstrated that the HABP exerts anti-tumor effect on NF1 cells. This HABP is a small peptide composed of 17 amino acids that was initially isolated from the horseshoe crab, also called **tachyplesin** (52). It has an amphipathic structure conferred by two anti-parallel β-sheets held rigidly in place by two disulfide bonds. This structure appears to be critical for its anti-tumor activity (53). However, the **initial** target of HABP/tachyplesin outside the cell surface is largely unknown.

To elucidate the mechanism by which HABP/tachyplesin (briefly tachyplesin) can inhibit the growth of tumor cells, we began by investigating protein that can binds to both HABP/tachyplesin and tumor cell surface to trigger the cell killing. Using the T7 phage display technique, we identified the C1q sub-component of human complement 1 as a potential **initial** target. The interaction between C1q and tachyplesin was confirmed by an ELISA and by affinity-precipitation plus Western blotting. These results

suggest that the binding of tachyplesin to C1q triggers the activation of classical complement pathway and leads to the killing of tumor cells. Interestingly, this effect was blocked if the target cells were pretreated with hyaluronidase or an excess of hyaluronan. This indicates that tachyplesin may initially target hyaluronan and related compounds on the cell surface and subsequently bind to C1q in the serum to activate the complement pathway to destroy the integrity of cell membrane leading to a cell killing cascade, in addition to its binding to Bcl-2 and Bcl-xL as to pro-apoptosis, its Blocking of phosphorylation of ERK and its inhibition of cyclin B1 and cdc2. It seems that the **anti-tumor effect of HABP/tachyplesin is exerted with multiple targets/pathways**, which is a preferred action mechanism for anti-tumor.

The following is summarized the evidence that HABP/tachyplesin act via triggering classical complement cell killing pathway.

1) Isolation and Characterization of Tachyplesin-binding Phages: To identify sequences that bind to tachyplesin, we screened a phage-displayed library of some 1.6×10^7 unique clones expressing sequences from tumor cells ranging in size from 300 to 3,000 base pairs in length fused to the T7 gene 10 capsid protein. Phage particles expressing tachyplesin-binding proteins/peptides were affinity purified on the wells of a microtiter plate coated with tachyplesin or control peptide. After 4 or 5 rounds of bio-panning, the number of phages from the tachyplesin-coated plates was approximately 100-fold greater than that from control peptide plates. Ten plaques were then selected and amplified by PCR. Eight had the same size PCR products and some of these were then sequenced. The deduced amino acid sequences were then subjected to a blast analysis and the results repeatedly identified human C1q, a subcomponent of complement. The region of C1q involved in the binding to tachyplesin was located around the first 400 base pairs of complement C1q B chain open reading frame (Genebank access number NM_000491), corresponding to the N-terminal collagen-like domain of C1q (54).

2) Binding of the C1q to Tachyplesin: To further test the possibility that tachyplesin binds to C1q, we examined the interaction between these two proteins using an ELISA-like system. In the first assay, plates were coated with tachyplesin or the control peptide, probed with C1q and then the amount of bound C1q was detected with anti-C1q.

As shown in **Fig. 16 A,** C1q binds to immobilized tachyplesin in a dose-dependent manner, but not to the control peptide. Similar results were obtained when the plates were pre-coated with C1q and then probed with biotinylated tachyplesin which was detected by a streptavidin coupled system (**Fig. 16 B**). However, this interaction was significantly reduced if the tachyplesin was denatured by reduction and alkylation of the disulfide bonds and further acetylation of the charged side chains (**Fig. 16 C**), which suggests that the interaction between tachyplesin and C1q depends upon the secondary structure of tachyplesin.

The binding of tachyplesin to C1q was also dependent on the NaCl concentration used in the assay buffer (**Fig. 16 D**). The maximum binding occurred at 0.15 M NaCl, the normal physiological salt concentration. Both increasing and decreasing the ionic strength in the assay drastically reduced the binding.

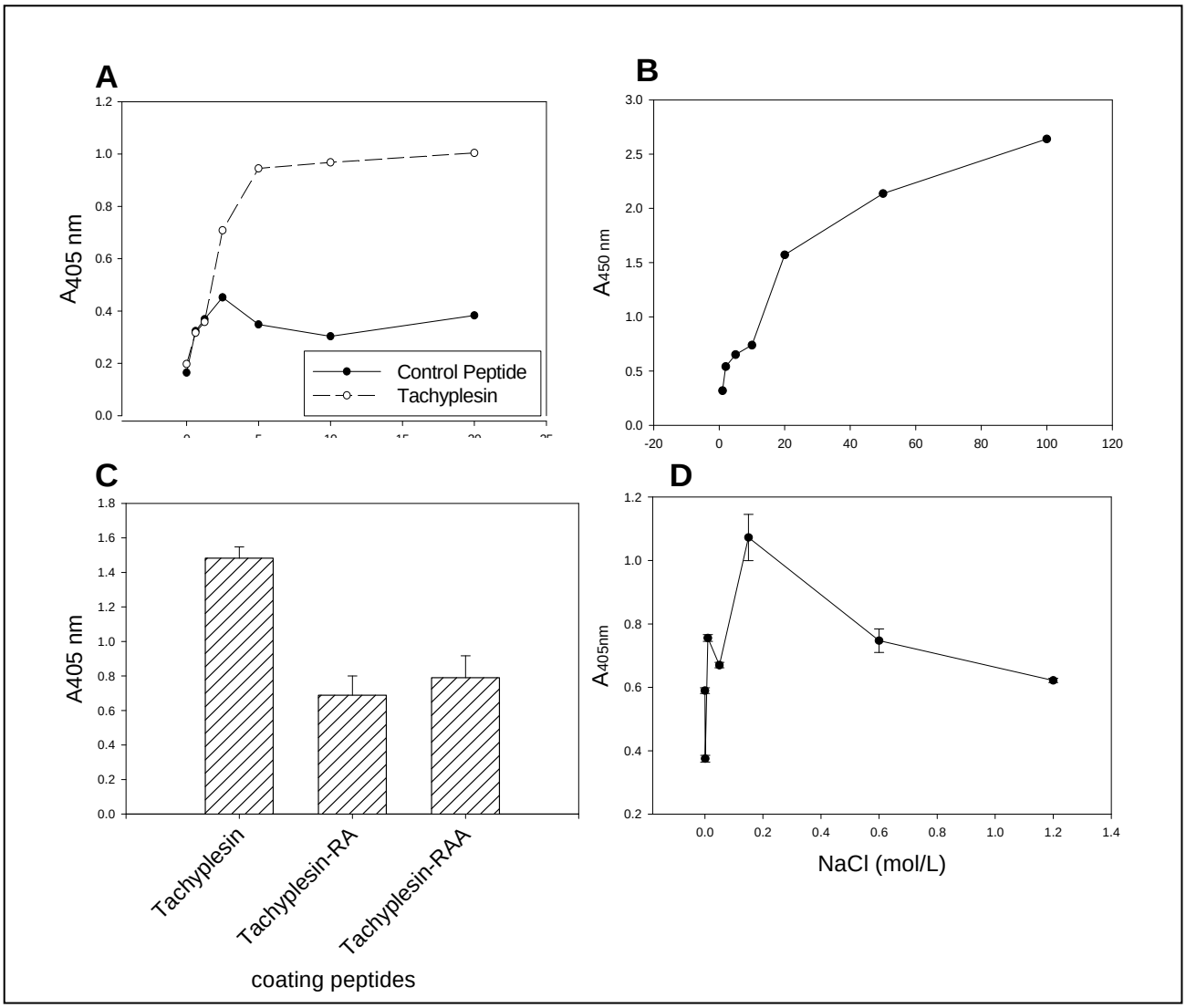


Fig. 16 The interaction between tachyplesin and the C1q subcomponent of complement 1. **A)** ELISA plates were coated with varying concentrations of either tachyplesin or the control peptide and then incubated with 1 μg of C1q that was then detected with anti-C1q and peroxidase conjugated detection system. The C1q bound to the plate coated with tachyplesin but not to that coated with the control peptide. **B)** Plates were coated with varying concentrations of purified C1q, probed with biotinylated tachyplesin (1 μg/ml) and then assessed with the streptavidin conjugated peroxidase and substrate system. Again, the tachyplesin bound to the immobilized C1q. **C)** Plates were coated with similar amounts (10 μg/ml) of native, reduced and alkylated (RA), and reduced, alkylated and acetylated (RAA) tachyplesin. Then, the plates were probed with 100 ng/ml of C1q followed by anti-C1q and peroxidase conjugated detection system. The binding of C1q to native tachyplesin was significantly greater than either of the denatured peptides. **D)** To test the effect of ionic strength on the interaction, ELISA plate coated with 10 μg/ml of C1q and then incubated with 0.1 μg/ml of biotinylated tachyplesin in VBS buffer of variable ionic strength. The maximum binding corresponded to physiological ionic strength of 0.15 M NaCl.

The interaction between tachyplesin and C1q was further examined by affinity-precipitation and Western blotting of normal human serum. For this, a biotinylated version of tachyplesin was incubated with normal human serum, followed by streptavidin-Sepharose. The immobilized proteins were then eluted and analyzed by Western blotting with anti-C1q polyclonal antibody. As shown in **Fig. 17**, probing the blot with

a poly-clonal antibody revealed three bands corresponding to three chains of the C1q complex (A 27.5, B 25.2, and C 23.8 kDa, respectively) in the tachyplesin treated sample, but not in the samples without the peptide or with the control peptide. Not surprisingly, only a small portion of C1q from the serum was pulled down since serum contains a relatively high concentration of this protein (80 μg/ml) (21). Taken together, these results suggests that C1q binds to both immobilized (surface-bound) and free (liquid-phase) tachyplesin, and tachyplesin binds to both purified and serum C1q, which confirms that there is true interaction between these two molecules.

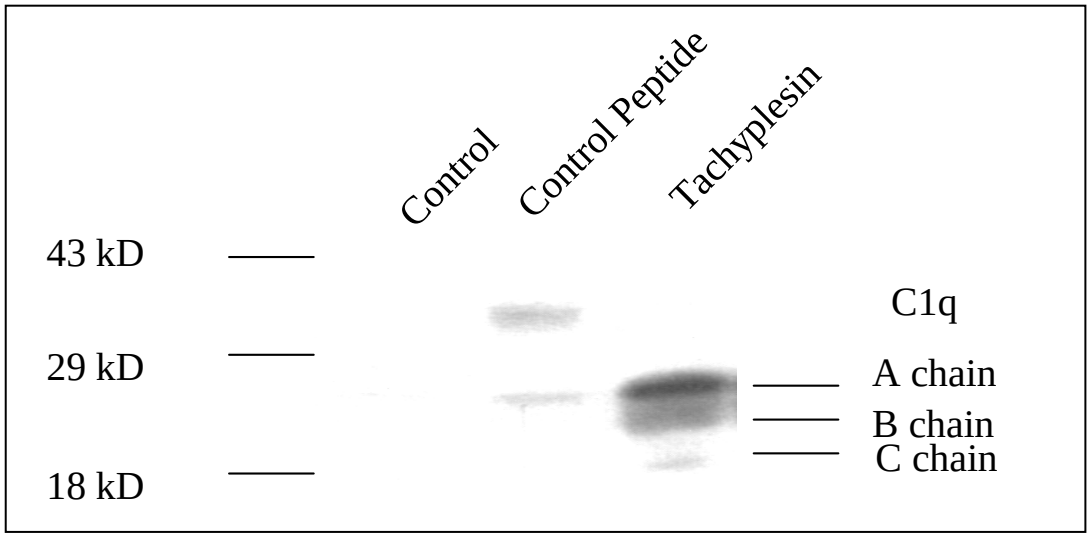


Fig. 17 Affinity-precipitation of serum C1q with tachyplesin. Normal human serum was incubated without or with biotinylated tachyplesin and control peptide, along with streptavidin-Sepharose in VSB buffer at 4 ºC overnight. After washing, the beads were extracted in Laemmli sample buffer (reducing conditions) and subjected to SDS-PAGE and Western blotting with anti-C1q antibody. Three bands representing the A, B, C chains of C1q (27,550, 25,200, 23,800 Dalton, respectively) were apparent in the sample incubated with biotinylated tachyplesin but not detected in the absence of peptide or in the control peptide. The results are representative of three different experiments.

3) Activation of the Classical Complement Pathway by HABP/Tachyplesin: To determine if tachyplesin could activate the complement pathway, we used ELISAs with tachyplesin-coated microplates. Normal serum was diluted with VBS (containing Ca^{2+}) and applied to wells coated with tachyplesin or the control peptide, washed and then probed with polyclonal antibodies against C4, C3, and C5b-9. As shown in **Fig. 18 A,** the significant amounts of activated fragments of C4b, C3b, and C5b-9 complex were detected in the forms of property. Furthermore, when the same fresh serum was heat-inactivated (56 ºC, 30 min) prior to the use, the activated fragments of C4b, C3b, and C5b-9 complex were detected at a level of background similar to the control peptide in the same ELISA. These data strongly demonstrates that tachyplesin is able to trigger the activation of whole classical complement cascade, since the appearance of C4b, C3b, and C5b-9 complex is the characteristics of the classical activation of complements.

To visualize the existing of activated C4b fragments, Western blotting was performed. Normal fresh human serum was mixed with the biotinylated tachyplesin or control peptide, affinity-precipitated with streptavidin-Sepharose, subjected to SDS-PAGE and finally Western blotted using antibodies to C4. **Figure 18 B** shows that the three peptide chains of C4b (α 97 kDa, β 75 kDa, and γ 33 kDa, respectively) were affinity-precipitated in the tachyplesin-treated sample, but not in those treated with the control peptide or in the absence of peptide. These results are consistent with the results obtained from ELISA, which further confirm that the tachyplesin is capable to activate the classical complement cascade, although we cannot

exclude the possibility that tachyplsein binds selectively and directly to C4b and then activate the down-stream molecules.

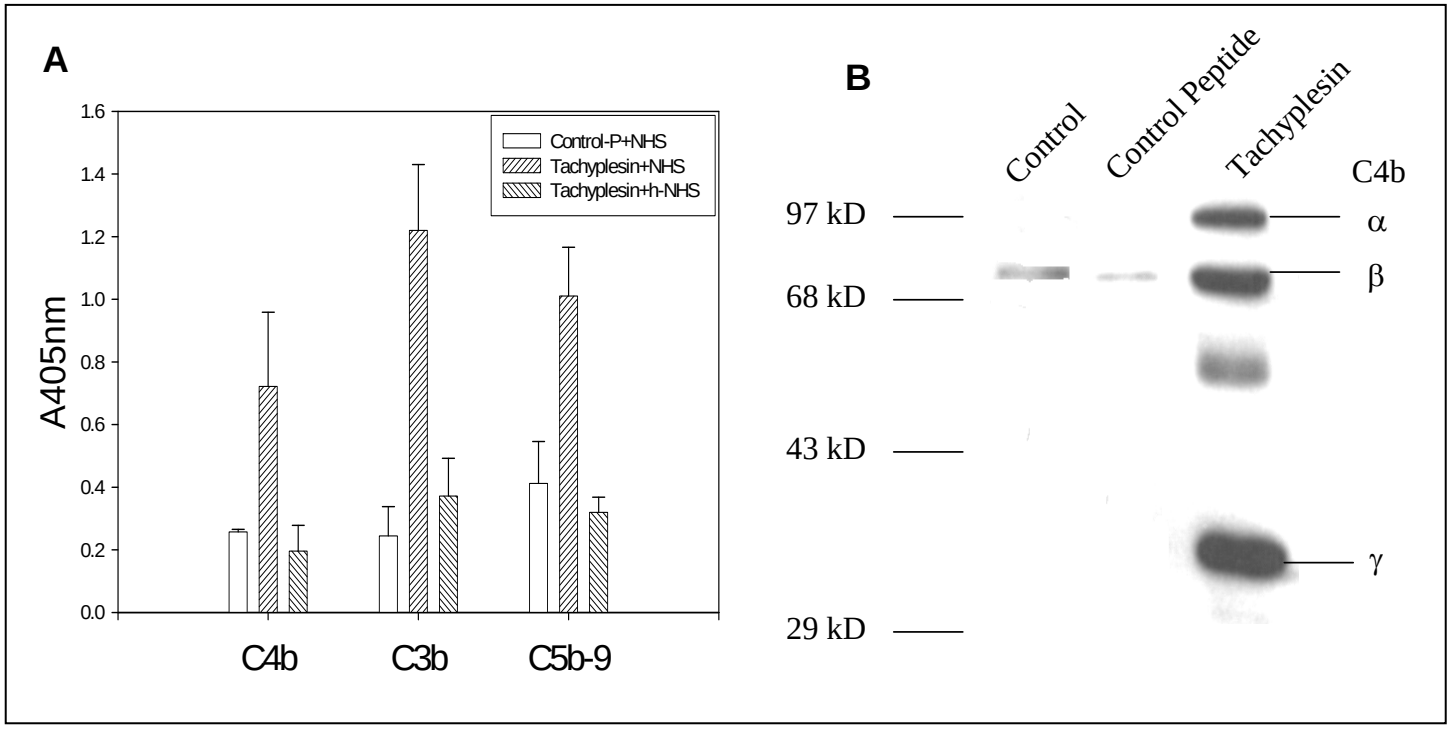


Fig. 18 Determination of tachyplesin-mediated activation of the classical complement pathway by ELISA and affinity-precipitation. A) Microtiter plates coated with either tachyplesin of the control peptide (10 μg/ml), incubated with normal human serum (NHS) without or with heat-inactivation (h-NHS). The serum was diluted with VBS (1:2 for C4b, C3b and 1:20 for C5b-9). Complement activation was then assessed using polyclonal antibodies against C4, C3 and polyclonal antibody to C5b-9. There was a significant amount of immobilized C4b, C3b and C5b-9 detected in the wells that were coated with tachyplesin and incubated with normal human serum, as compared to those coated with the control peptide or incubated with heat-inactivated serum. **B)** For affinity-precipitation and Western blotting, normal human serum was treated without peptide or biotinylated control peptide or biotinylated tachyplesin. Streptavidin-Sepharose beads were added to the mixture, washed thoroughly and processed for SDS-PAGE and Western blotting with antibodies to C4b. The bands of α, β, γ chains of C4 (*Mr* 97, 75, and 33 kDa, respectively) were apparent in the samples treated with tachyplesin, but not with the control peptide or in the absence of peptide.

4) Role of hyaluronan in the deposition of C4b and C3b on Tumor Cells: Since tachyplesin contains a hyaluronan binding motifs [B(X)$_7$B] (55), we investigated the possibility that tachyplesin can bind to hyaluronan (both free and cell-associated). To this end, we took advantage of the fact that hyaluronan by itself does not bind to nitrocellulose but will do so in the presence proteins or peptides that bind to it (56). In the assay, tachyplsein was mixed with [^{3}H]-hyaluronan and then applied to a nitrocellulose membrane. The free [^{3}H]-hyaluronan was washed away with PBS, and the complex of [^{3}H]-hyaluronan-tachyplesin that was retained on the filter membrane was analyzed. **Figure 19 A** shows that tachyplsein binds strongly to hyaluronan, and this could be abolished by a 100-fold excess of unlabeled hyaluronan. In contrast, the control peptide showed little or no binding to [^{3}H]-hyaluronan, indicating that the binding of tachyplsein to hyaluronan was specific.

We then examined the binding of FITC-tachyplesin to tumor cells that express large amounts of hyaluronan on their surfaces. As shown in **Fig. 19 B**, tachyplsein was distributed on the surface of the cells. This binding was significantly reduced by the addition of an excess of free hyaluronan on pre-treatment with

hyaluronidase (**Fig 19 C**) as demonstrated by flow cytometer analysis. These results suggest that hyaluronan or related molecules such as chondroitin sulfate act targets for tachyplsein on the cell surface.

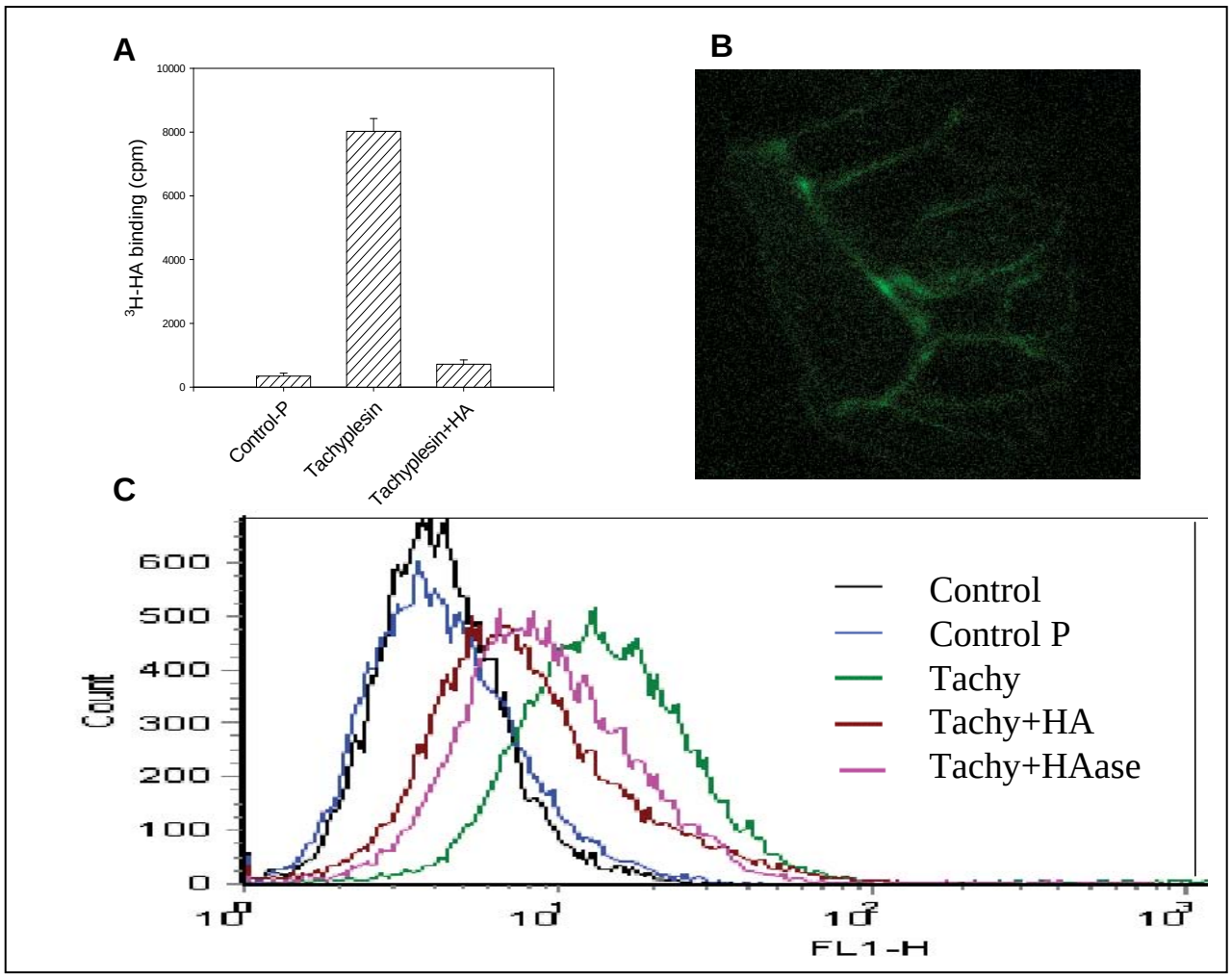


Fig. 19 Hyaluronan-mediated attachment of tachyplesin to TUMOR tumor cells. A) To determine the binding of tachyplesin to HA, tachyplesin and the control peptide were mixed with [^{3}H]-HA, applied to a sheet of nitrocellulose and extensively washed. A significant amount of [^{3}H]-HA bound to the nitrocellulose in the presence of tachyplesin but not in the control peptide or with an excess of unlabeled HA. **B)** One million tumor cells were incubated with 1 μg/ml of FITC-tachyplesin at 4 ºC for 30 min, fixed with freshly prepared 4% formaldehyde and analyzed with confocal microscopy. Tachyplesin was associated with the surfaces of the cells. **C)** To determine the role of cell surface HA in the attachment of tachyplesin to tumor cells, the tumor cells were incubated with or without hyaluronidase (0.1 mg/ml) at 37 ºC for 1 hour. Then, 10 μg/ml of FITC-tachyplesin was added to the cells at 4 ºC for 30 min followed by fixation. Flow cytometry showed that the binding of FITC-tachyplesin to the cell surface was partially blocked by pretreatment with hyaluronidase or an excess of HA.

In the classical pathway of complement activation, 4-to 5-fold more C3b than C4b is deposited on the surfaces of target cells. In addition, C3 contains a thioester moiety that can form covalently bonds with nearby molecules in the transition from C3 to C3b. Thus, C3b deposition represents an index of complement activation. For this reason, we tested whether tachyplsein could induce the deposition of C3b on tumor cells. Tumor cells were incubated in a mixture of normal serum and tachyplesin, stained with antibodies to C3 then examined by confocal microscopy. As shown in **Fig. 20A and B**, C3b was indeed deposited on the surfaces of tumor cells.

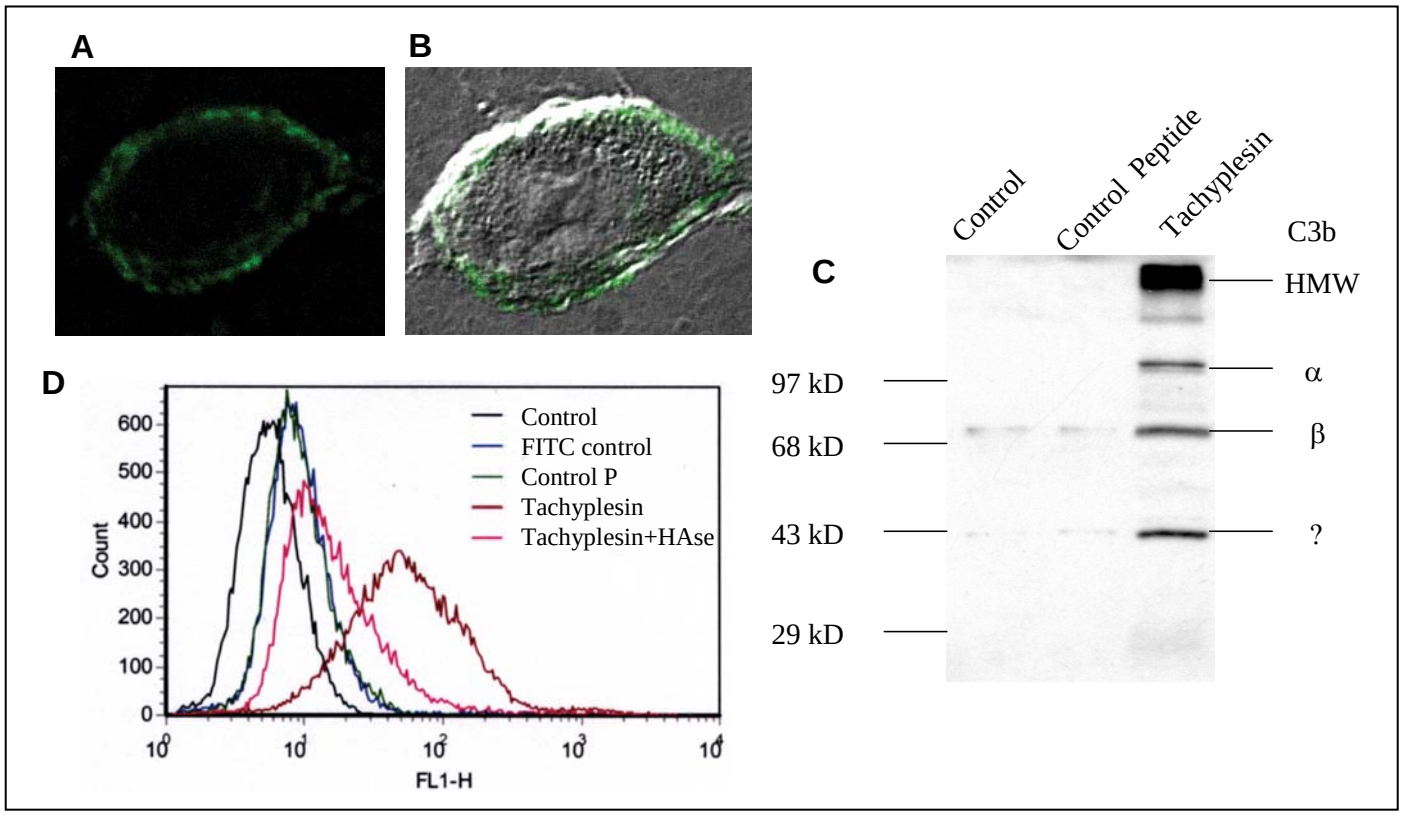


Fig. 20 Effect of tachyplesion on the induction of complement on the surfaces of tumor cells. A and B) tumor cells were incubated with tachyplesin in the presence of 10% normal human serum for 20 min, washed and then incubated with a polyclonal antibody to C3 (4 ºC for 30 min) followed by a FITC-secondary antibody and analysis confocal microscopy. Both immunoflourescence alone and the merger of fluorescence and transmitted images are shown. **C)** To analyze complement activation by Western blotting, TSU cells were incubated in 10% normal human serum in the presence or absence of either tachyplesin or control peptide at 37 ºC for 60 min. After washing, the cell lysates were harvested and processed for Western blotting with antibodies to C3b. In the cells treated with tachyplesin, there were two bands corresponding to α and β subunits of C3b (115 and 75 kDa), as wells as a high molecular weight band (HMW) representing C4b covalently bound to membrane constituents. These bands were reduced in cells treated without peptide or with the control peptide. **D)** To examine the role of cell surface HA in the deposition of complement, 100 μg/ml peptides were added to the cells culture in the presence of 10% normal human serum and incubated for 20 min at room temperature. The cells were then washed, incubated with a goat polyclonal antibody to human C3 (20 μg/ml) at 4 ºC for 30 min followed by incubation with FITC-conjugated anti-goat IgG (1:200) at 4 ºC for 30 min. The cells were finally stained with propidium iodide and analyzed with FACS. Preincubation with hyaluronidase abrogated the C3 deposition on tumor cell surfaces.

The presence of activated C3b was also demonstrated by Western blotting of tumor cells following treatment with serum and tachyplesin. **Figure 20 C** shows that in the samples treated with tachyplesin, two subunits of C3b (α 115 kDa and β 75 kDa) and possibly degraded iC3b (? band for α1). Significantly, a high molecular weight band (HMW in **Fig. 20 C**) was found with Western blotting under reducing conditions, indicating a covalent linkage to large membrane constituents. Scans of these Western blots revealed that the majority of deposited C3b (70% to 80%) was present in this high molecular weight form. This is consistent with the fact that in the transition from C3 to C3b, a thioester moiety can form covalently bonds with nearby molecules. FACS analysis of cells treated with tachyplsein and serum (**Fig. 20 D**) showed that there was a significant increase in FITC-tagged antibody to C3, indicating that C3b deposition on the tumor cells. This did not occur with cells treated with the control peptide. These results are consistent with those from confocal microscopy and Western blotting.

Since tachyplsein can bind to hyaluronan, we investigated the possibility that membrane-bound hyaluronan plays a role the binding of tachyplesin-mediated activation of complement on the surface of tumor cells. The suspensions of tumor cells ere pretreated with hyaluronidase before the addition of tachyplsein and human serum, and the presence of C3b was detected immuno-staining followed by FACS analysis. As shown in **Figure 20D**, hyaluronidase pre-treatment markedly reduced the intensity of fluorescence, indicating a reduction in C3b deposition. Thus, hyaluronan or related glycosaminoglycans do appear to play a key role in the activation of complement on the cell surface by tachyplesin.

5) Effect of tachyplsein on Tumor Cells: The fact that tachyplsein can trigger the deposition of complement on the surfaces of tumor cells via classical pathway, suggesting that it might kill cells by disrupting the integrity of the plasma membrane. To test this possibility, we examined the permeability of cell membrane with macromolecule FITC-Dextran, which is excluded by the membranes of viable, healthy cells but can pass through the damaged plasma membrane of unhealthy cells (2). **Figure 21A** showed that when cells were treated with tachyplsein and human serum, the fluorescence spectrum was shifted, indicating that more FITC-Dextran had passed through the plasma membrane and entered the cytoplasm. Thus, it appears that treatment with tachyplsein disrupted the cell membrane and increased its permeability.

Finally we examined the effect of tachyplsein and normal human serum on cell growth. As indicated in **Figure 21B**, tumor cells treated with tachyplsein showed a marked inhibition of proliferation in the presence of complete human serum. However, treatment of the cells with hyaluronidase significantly reversed this effect, again suggesting that cell-surface hyaluronan plays a critical role in tachyplesin-induced inhibition of tumor cell growth. Significantly, heat-inactivated serum also attenuated the effects of tachyplesin, but to a lesser extent than hyaluronidase treatment. These results imply that tachyplsein may have multiple effects on the cells leading to both growth arrest and death.

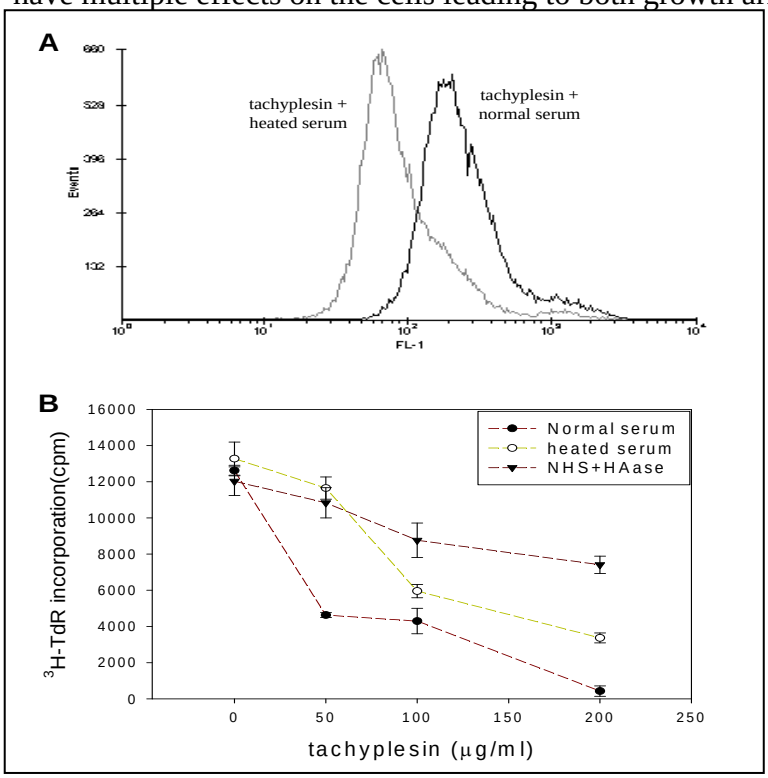


Fig. 21 Biological Effect of hyaluronidase on tachyplesin-mediated cytotoxicity of tumor cells. A) To determine whether tachyplesin damages the plasma membrane, TSU cells were incubated with tachyplesin plus or heat-inactivated human serum or normal human serum (NHS) overnight. After harvesting, the cells were incubated with 5 μg/ml of FITC-Dextran (*Mr* 40,000) and subject to flow cytometry. The amount of FITC-Dextran taken up by the cells was significantly higher in those treated with tachyplesin than in the control cells, suggesting that tachyplesin disrupts the integrity of the plasma membrane. **B)** Tumor cells were treated with hyaluronidase (0.1 mg/ml) at 37 ºC for 1 hour. Then, tachyplesin and 2% normal human serum or heat-inactivated serum were added and incubated overnight followed by a [^{3}H]-thymidine incorporation assay. The cytotoxic effects of tachyplesin in present of normal serum were reduced by either treatment with hyaluronidase or heat-inactivated serum.

The HABP / tachyplesin binding to both hyaluronan (HA) on the cell surface and C1q in the serum and activate the classical complement cascade would damages the integrity of the membranes of the tumor cells. To prove this, the death signal triggered from external apoptotic stimulus through FADD path was

examined. After the STS26T cells were treated with HABP / tachyplesin at dose of 0, 50 or 100 ug/ml for 24, 48 or 72 hr, the cell lysate was harvested and assayed with Western blotting and the activities of Caspase 8 and Caspase 3 with fluorescence substrate assay. The results showed that the binding of HABP / tachyplesin to HA on the cell surface and C1q in serum did trigger the external apoptotic stimulus through FADD path as evidenced by the increased FADD, cleaved Caspase 8, 3 and PARP (**Fig 22A**) and enhanced activities of Caspase 8 and 3 (**Fig 22B and C**).

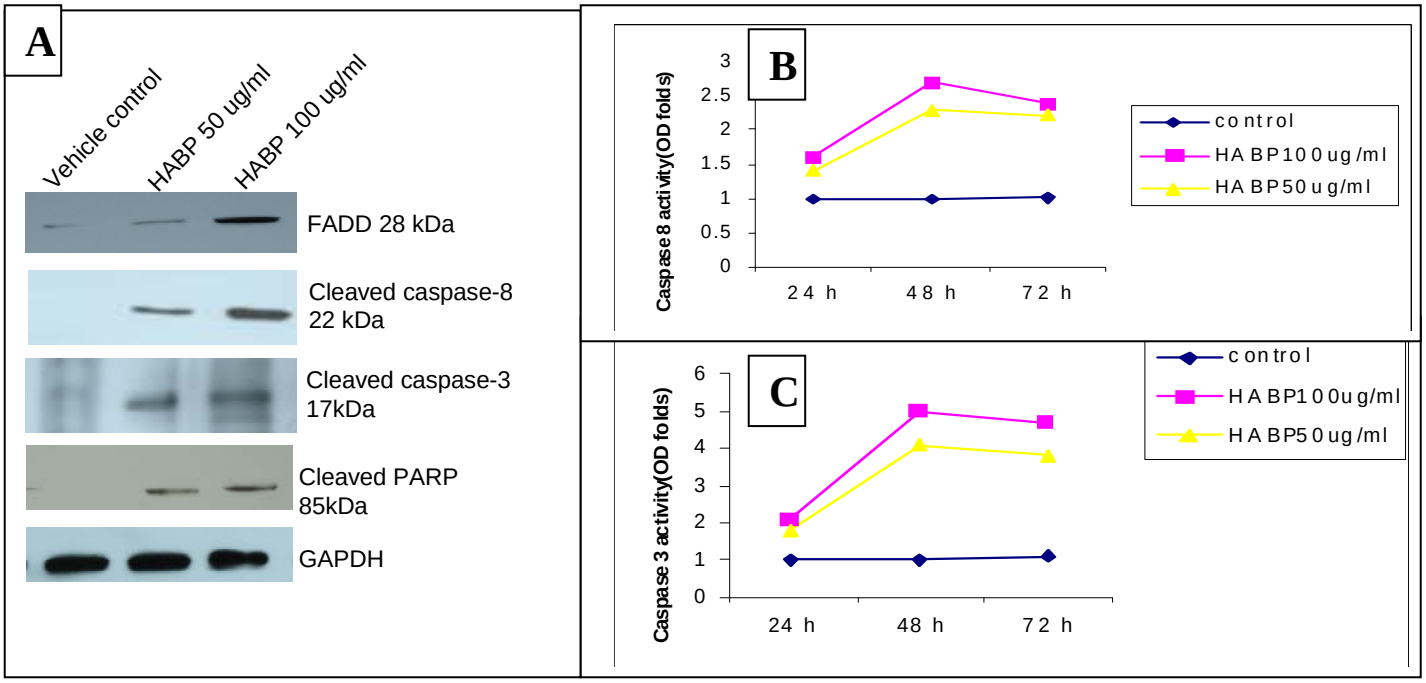


Fig 22. Binding of HABP / tachyplesin to HA on the cell surface and C1q in serum triggered the external apoptotic stimulus through FADD path. After the STS26T cells were treated with HABP / tachyplesin at dose of 0, 50 or 100 ug/ml for 24, 48 or 72 hr, the cell lysate was harvested and assayed with Western blotting (**A**) and the activities of Caspase 8 (**B**) and Caspase 3 (**C**) with fluorescence substrate assay.

Taken together, these observations suggest that HABP / tachyplesin bind to both hyaluronan on the cell surface and C1q in the serum and activate the classical complement cascade, which damages the integrity of the membranes of the tumor cells, triggers the external apoptotic stimulus through FADD path, resulting in the death of NF tumor cells.

4. Effect of HABP on NF tumor in mice model

After intensive study of underlying mechanism of anti-tumor effect of HABP, we examined if the in vitro anti-tumor effect can be translated in vivo.

In vitro, the ST88-14 cells grow faster than other two cell lines (NF90-8 and NF88-3) derived from patients of neurofibromatosis. However, when we subcutaneously injected 2.5 x 10^6 ST88-14 cells into nude mice, we did not observe the growth of tumor. We speculated that this might due to insufficiency of cells injected. Then, we injected 10^7 ST88-14 cells, but the tumor was still not formed.

To increase the possibility of forming tumor exnograft in mouse, we decided to try SCID (severe combined immunodeficient) mice that defect in both T lymphocytes and B lymphocytes. Compared with the nude mice that only defect in T lymphocytes, the SCID mice confer a less capability to reject the implanted tumor cells. However, to our dismay, the 10^7 ST88-14 cells still could not form tumor in SCID mice.

We then tired to implant the NF1 cells in organs that have a less extent of immune surveillance and more extent of the supply of nutrition, such as liver. Five million of ST88-14 cells were injected into sub-capsule of liver. Still, to our disappointment, the tumor nodule did not form.

Since the growth of NF1 tumor in mice is prerequisite for test the effect of HABP *in vivo*, we were actively seeking for the advice from experts in this field. As the result of this effort, we found that the STS26T cells has an extremely high level of DcR3, a decoy death receptor that could bind to Fas L, VEGI and LIGHT, block apoptosis signal and enhance the survival of STS26T cells. 5 x 10^6 STS26T cells were subcutaneously injected into nude mice (5 /group), allowed tumor to 50 mm3 and treated with vehicle alone (as control) or HABP 20 mg/kg i.p every day for 6 weeks. The size of tumors were measured every 4-5 days and tumor were weighted at the end of experiment. The results were very promising. The NF tumor treated with HABP grew much slower than vehicle control (**Fig 23A**) and tumors were much smaller than that in control (**Fig 23B**). The in situ TENUL assay showed that the HABP treated tumors had more apoptotic body than the control (**Fig 23C**) with statistical significance (**Fig 23D**).

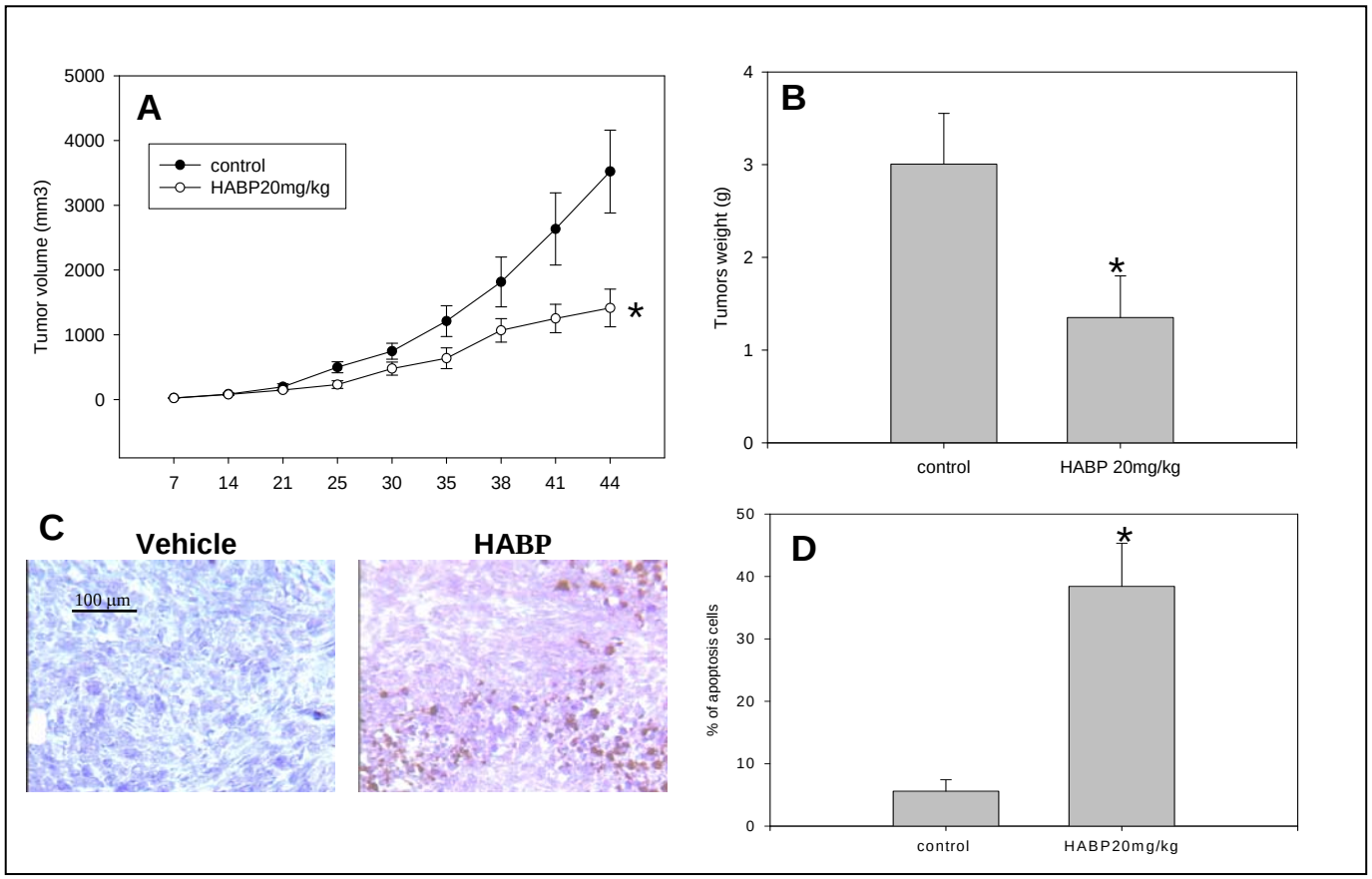


Fig 23. Anti-tumor effect of HABP / tachyplesin on NF tumor formed by STS26T in nude mice. 5 x 10^6 STS26T cells were subcutaneously injected into nude mice (5 /group), allowed tumor to 50 mm3 and treated with vehicle alone (as control) or HABP 20 mg/kg i.p every day for 6 weeks. The sizes of tumors were measured every 4-5 days and tumors were weighted at the end of experiment.

In summary, in the funding period we have carried out lager amount of experience to determine the anti-tumor effect of HABP and its underlying mechanism. We have finished the following main tasks as proposed in application and performed more experiments than what we proposed. It is a successful project which provides a lot of new information about the anti-tumor effect of HABP and NF biology. Since the HABP /tachyplesin is a simple biological peptide without any toxicity, it might have the potential to be used in anti-NF. It is worth to further pursue this project.

Key Research Accomplishments

We have accomplished the follow tasks:

1) Chemically synthesize HABP and control peptide in a large scale;

2) Identify its HA binding activity;

3) Study the effect of HABP on phosphorylation of ERK1**,** the level of cell cycle related molecules, such as cyclin B1 and cdc 2;

4) Study the effect of HABP on Bcl-2

5) Isolate and characterize the HABP/tachyplesin-binding phages**;**

6) Define the initial binding of the C1q to HABP/tachyplesin;

7) Elucidate the activation of the classical complement pathway by HABP/tachyplesin;

8) Define the role of hyaluronan in the deposition of C4b and C3b on tumor cells;

9) Study the effect of HABP on molecules involved in apoptosis of ST88-14 and STS26T NF cells;

10) Characterize the anti-tumor activity of HABP *in vivo*.

Conclusions

- HABP/tachyplesin can be chemically synthesized in a large scale.
- The synthetic HA binding peptide possesses its bioactivity of binding to HA.
- HABP can significantly change the morphology of treated ST88-14 and STS26T NF cells and inhibit the proliferation or colony formation of ST88-14 and/or STS26T NF cells in a dose-dependent manner in both an anchorage-dependent and anchorage-independent conditions.
- HA binding peptide binds to Bcl-2/Bcl-x_L and induces apoptosis, which may be one of the mechanisms by which HABP inhibits ST88-14 and STS26T NF cells.
- HA binding peptide is capable of reducing the level of phosphorylated ERK1.
- HABP reduces the level of cell cycle related molecules, such as cyclin B1 and cdc 2.
- HABP/tachyplesin binds to C1q on cell surface, activate the classical complement cascade, since it triggered several down-stream events including the cleavage and deposition of C4 and C3 and the formation of C5b-9. which can be blocked if the tumor cells are treated with hyaluronidase or a large excess of hyaluronan, indicating that hyaluronan or related glycosaminoglycans were involved in this process.
- The combination of HABP/tachyplesin and human serum can markedly inhibit the proliferation of tumor cells and this effect is attenuate if the serum is heat-inactivated or if hyaluronidase is added.
- The HABP/tachyplesin exerts anti-tumor effect on NF cells both *in vitro* and *in vivo.*

Reportable outcomes
(Due to or partially due to this support)

Papers

1. Xue-Ming Xu, Yixin Chen, Feng Gao, Jinguo Chen, Shanmin Yang, Charles B. Underhill and Lurong Zhang: A Peptide with Three Hyaluronan Binding Motifs Inhibits Tumor Growth by Inducing Apoptosis. Cancer Res. 2003; 63(18):5685-90.

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4. Jinguo Chen, Xue-Ming Xu, Charles B. Underhill, Shanming Yang, Luping Wang, Yixin Chen, Shuigen Hong, Karen Creswell, Lurong Zhang: Tachyplesin Activates the Classical Complement Pathway to Kill Tumor Cells. Cancer Res. 2005; 65(11):4614-22.

Abstracts

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